

Inhibitors of the *Yersinia* protein tyrosine phosphatase through high throughput and virtual screening approaches

Xin Hu^{a,†}, Milos Vujanac^{b,†}, Noel Southall^a, C. Erec Stebbins^{b,*}

^a NIH Chemical Genomics Center, National Center for Advancing Translational Sciences, National Institutes of Health, 9800 Medical Center Drive, Rockville, MD 20850, United States

^b Laboratory of Structural Microbiology, The Rockefeller University, New York, NY 10065, United States

ARTICLE INFO

Article history:

Received 12 September 2012

Revised 4 December 2012

Accepted 10 December 2012

Available online 20 December 2012

Keywords:

Yersinia virulence

YopH

High throughput screening

Virtual screening

ABSTRACT

The bacterial protein tyrosine phosphatase YopH is an essential virulence determinant in *Yersinia pestis* and a potential antibacterial drug target. Here we report our studies of screening for small molecule inhibitors of YopH using both high throughput and in silico approaches. The identified inhibitors represent a diversity of chemotypes and novel pTyr mimetics, providing a starting point for further development and fragment-based design of multi-site binding inhibitors. We demonstrate that the applications of high throughput and virtual screening, when guided by structural binding mode analysis, is an effective approach for identifying potent and selective inhibitors of YopH and other protein phosphatases for rational drug design.

© 2012 Elsevier Ltd. All rights reserved.

Infection with *Yersinia* spp. has represented one of the greatest human health concerns, causing human diseases ranging from gastrointestinal syndromes to Bubonic Plague.¹ *Yersinia pestis*, the causative agent of the plague, is classified as a Category 'A' bioterrorism hazard by the Centers for Disease Control. Plague killed roughly one-third of the European population in the middle ages, and has been estimated to have taken the lives of over 200 million people worldwide.² While effective therapies exist, treatment of these pathogenic bacteria has become more difficult in the last decades due to the emergence of antibiotic resistance. Such bacteria have gained resistance to common medicines such as penicillin, chloramphenicol, and tetracycline. In addition, there is increasing risk of misuse of infectious agents as weapons of terror, as well as instruments of warfare for mass destruction.³ Therefore, the development of novel treatments of these bacterial infections has taken on a new urgency.

The *Yersinia* genus itself consists of three members, *Yersinia pestis*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis*. *Y. pestis* is the causative agent of the plague, whereas *Y. enterocolitica* and *Y. pseudotuberculosis* typically cause a food borne illness. These pathogens utilize a Type 3 Secretion System (T3SS) to translocate six virulence proteins into the cytosol of host cells.⁴ These *Yersinia* effectors, which are referred to as Yops (*Yersinia* outer proteins), include YopH, YopE, YopJ/P, YpkA/YopO, YopT, and YopM. YopH, which belongs to the class of enzymes called protein tyrosine

phosphatases (PTPs), is an essential virulence factor that plays a key role in *Yersinia* pathogenicity.⁵ As a potential drug target, YopH has attracted attention in the search for effective antibacterial candidates to combat *Yersinia* virulence. Efforts in the development of small molecule inhibitors of YopH have led to the discovery of promising lead compounds.^{6–12} Recently, several crystal structures of YopH in complex with small molecule inhibitors have been determined,^{13–16} offering promise in structure-based design of potent and selective compounds against YopH for drug development.

We previously reported the discovery of small molecule leads against the *Yersinia* protein kinase A (YpkA) using in silico database screening.¹⁷ Aimed at identifying structural features of YopH for structure-based drug design, we have also performed a comparative docking study of YopH with respect to the *Salmonella* PTP (called SptP) and the eukaryotic PTP1B,¹⁸ and applied molecule docking and 3D-QSAR approach to investigate the probable binding interactions of two series of inhibitors of YopH: α -ketocarboxylic acid and squaric acid.¹⁹ Herein, we report our efforts in finding novel inhibitors of YopH through a combination of high throughput screening (HTS) and virtual screening (VS).

HTS was carried out at the Rockefeller University HTS Facility for a set of ChemDiv library containing 15,000 drug-like compounds. The compounds were screened in a 96-well format through an in vitro assay using the catalytic domain of YopH (residues 163–468) and pNPP as substrate. The inhibitory activity of the compounds was determined in comparison to 200 μ M sodium orthovanadate, a general PTP inhibitor. Compounds displaying significant inhibition at 100 μ M were subjected to further analysis. Of 49 initial hits, 15 compounds were selected for closer

* Corresponding author. Tel.: +1 (212) 327 7190; fax: +1 (212) 327 7191.

E-mail address: stebbins@rockefeller.edu (C. Erec Stebbins).

[†] These authors contributed equally to this study.

Table 1

Inhibitors of YopH identified through HTS and VS

Compound	Structure	IC ₅₀ (μM)	Screen method	Predicted binding mode
1		0.38	VS	U
2		0.98	VS	U
3		1.22	VS	I
4		1.68	VS	I
5		1.89	VS	U
6		1.92	VS	I
7		2.15	VS	Y
8		2.35	VS	Y

(continued on next page)

Table 1 (continued)

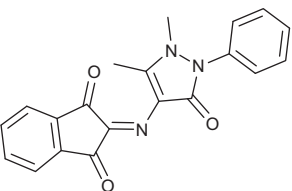
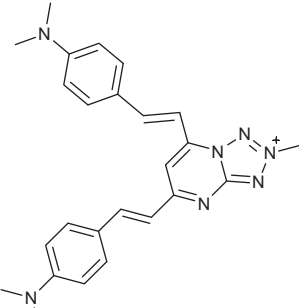
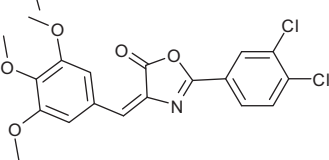
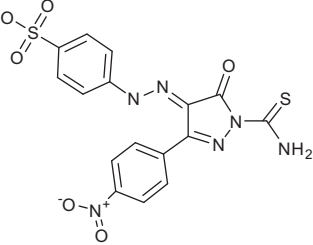
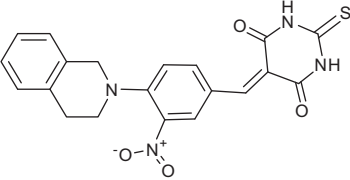
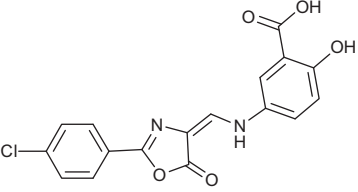
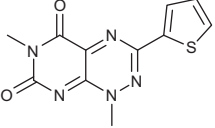
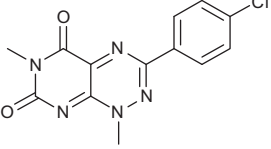
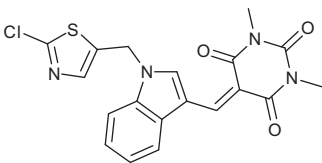
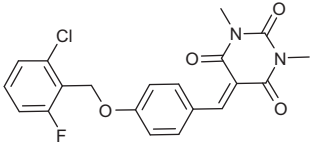
Compound	Structure	IC ₅₀ (μM)	Screen method	Predicted binding mode
9		4.01	VS	I
10		4.44	VS	Y
11		4.48	VS	I
12		4.61	VS	Y
13		4.73	VS	U
14		8.10	VS	U
15		0.88	HTS	Open
16		1.28	HTS	Open

Table 1 (continued)

Compound	Structure	IC ₅₀ (μM)	Screen method	Predicted binding mode
17		4.61	HTS	Open
18		9.89	HTS	Open

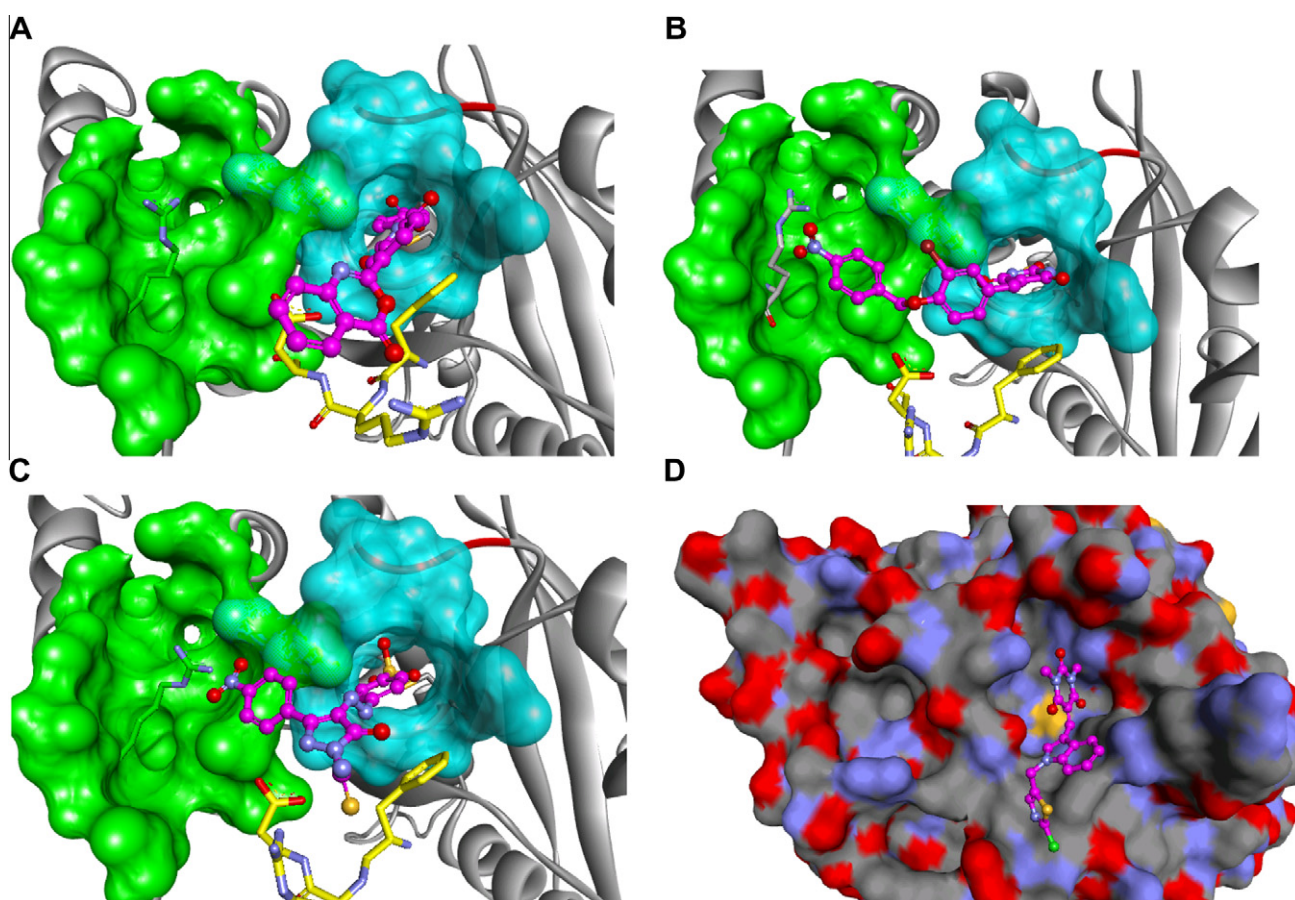
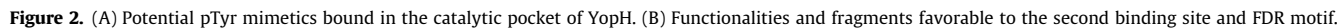


Figure 1. Predicted binding mode of YopH inhibitor (A) 'I-shaped' binding mode with compound **6**, (B) 'U-shaped' binding mode with compound **5**, (C) 'Y-shaped' binding mode with compound **12**, (D) open binding mode with compound **17**. The pTyr catalytic binding pocket is shown with surface in cyan color. The secondary phosphate binding site is shown with surface in green color and residue Arg205 in the pocket is shown with sticks. The conserved FDR (Phe229-Arg230-Asp231) motif of YopH is shown with sticks in yellow color of carbene atom and the nitrogen and oxygen are in blue and red color.

inspection and IC₅₀ determination. When interrogating IC₅₀ values, we used two known specific YopH inhibitors, aurintricarboxylic acid (ATA) and furanyl salicylate, as controls. They both produced IC₅₀ values similar to previously reported results.^{7,12} In parallel with HTS, a large-scale virtual screening was performed for the entire ChemDiv library containing more than 400,000 drug-like compounds. The crystal structure of YopH in complex with small molecule inhibitor nitrophenyl phosphate (PDB 1PA9) was used to dock compounds into the active site.¹⁴ To improve the docking accuracy and screen efficiency we applied a two-step hierarchical

screening strategy using both FlexX and AutoDock.¹⁷ The top scored hits were visually inspected, and 85 compounds were cherry-picked for in vitro inhibitory activity assay.

The identified inhibitors from the two screening campaigns are summarized in Table 1. Of 85 tested hits from VS, 14 compounds had potency of IC₅₀ below 10 μM. The most potent hit is a salicylic acid derivative which has been extensively studied as inhibitors of YopH.^{9,12} These compounds showed a diversity of structural characteristics with the pTyr mimetics such as phosphonate, sulfonate, sulfonamide, carboxylate, benzoxylate, and salicylate that are



sess a core of pyrimidotriazine-dione, which likely acts as a pTyr mimetic bound in the catalytic pocket. Though these inhibitors are relatively small, they exhibited high potency compared to other

inhibitors from VS (IC_{50} = 0.88 μ M for compound **15**). Another series of compounds, **17** and **18**, share a common scaffold of dimethylpyrimidine-trione, similar to compound **4** and **9** identified from VS. Interestingly, the two chemotypes were not found in VS.

We attempted to determine the binding mode of the inhibitors by co-crystallizing them with protein, but have not yet obtained adequate crystals. We therefore performed a refined docking analysis and MD simulations to examine the binding interactions of the hit compounds to the binding sites of YopH. Overall, three binding modes were identified with these YopH inhibitors. The first and most common binding mode involved a common functionality binding in the conserved catalytic pocket to mimic the substrate phosphotyrosine, forming extensive H-bonding interactions with residues in the conserved P-loop and the flexible WPD loop. Figure 1A illustrates the binding interactions of compound **6**, the benzoic acid acting as a pTyr mimetic inserted into the catalytic pocket, whereas the benzoxazin-4-one group on the other end of the molecule was pointed to the conserved FDR (Phe229–Arg230–Asp231) motif in the front of the pocket. Such a straight 'I-shaped' binding mode is typically observed with inhibitors of YopH and PTP1B in crystal structure complexes. The second mode adopted a 'U-shaped' binding conformation that the compound occupied both the catalytic pocket and the secondary phosphate binding site (Fig. 1B). Compound **1**, the most potent hit identified in our screen, adopted the same binding mode. Our binding mode analysis showed that the high potency of this compound was apparently contributed from the characterized hydroxybenzoic acid in the pTyr binding pocket and the oxadiazolpyrazine group bound in the secondary phosphate binding site. Indeed, MD simulations of the binding complex indicated that the heterocyclic ring together with the fluorophenyl group were accommodated well in the secondary binding pocket and formed extensive hydrophobic and H-bonding interactions mainly with residue Arg205.

The third binding mode probably represents the most intriguing class of YopH inhibitors discovered. In this mode, in addition to the pTyr mimetic binding in the catalytic pocket, two functional arms occupy both the secondary phosphate binding site and the FDR site simultaneously. Compounds **7**, **8**, **10**, and **12** showed such a potential 'Y-shaped' binding conformation to YopH (Fig. 1C). However, compared to the hits in other two binding modes, these tri-site binding inhibitors did not exhibit higher potency as expected. Selected from database screening, these compounds are typically larger than required for shape complementary to each of sites, so it is not surprising that they are not optimal for the binding to YopH. Further structure-based lead optimization could improve the activity of these inhibitors.

Finally, we focused on the two series of inhibitors from HTS and investigated why they failed in VS. The binding scores of these docked compounds in VS were typically low as compared to the top-ranked hits. Docking studies showed that the pTyr mimetic of dimethylpyrimidine-trione was unable to bind into the catalytic binding pocket of YopH which was used in the VS. The WPD loop appeared to pose a significant steric hindrance to the bulky group. On the other hand, these inhibitors were accommodated well in the open conformation of the pTyr binding pocket of YopH (PDB 1YPT) (Fig. 1D), similar to the 'open-bound' PTP1B inhibitor of naphthyloxamic acid.²⁰ This inspired us to perform in silico screening using an open conformation of the WPD loop of YopH.

To investigate the selectivity of these identified YopH inhibitors we performed a comparative analysis with two other protein tyrosine phosphatases, eukaryotic PTP1B and Salmonella SptP.¹⁸ We modeled the binding of the compounds with these PTPases using an approach combining step-wise ensemble-docking with MM-PBSA calculations.²¹ In general, these YopH inhibitors showed weak binding affinities to SptP, but were more comparable to the binding of PTP1B, particularly for the inhibitors with 'I-shaped'

binding conformation (Supplementary data S1). The results were in agreement with our previous analysis that YopH and PTP1B shared similar binding interactions at both the active site and adjacent peripheral sites including a conserved YRD motif, whereas SptP exhibits large difference at these binding sites in terms of molecular surface shape and charge distribution.¹⁸ Notably, the 'U-shaped' compounds typically showed high binding affinities to YopH, suggesting that the selectivity of these YopH inhibitors were mainly contributed from the binding interactions at the second phosphate binding site.

The promiscuous inhibition of protein tyrosine phosphatases remains a major problem in the search of selective PTPase inhibitors for drug development. To probe the promiscuity of the current hits, we assessed the cross-activity of these compounds that have been tested in other biological assays. A promiscuity index (PI) was obtained by inspecting the assays in which the compound was tested and in how many of those assays it was reported to be active in PubChem. For inhibitors without any test results in PubChem, we searched for analogues and calculated the PI of the closest one to access the potential promiscuity of the structural scaffold. Overall, most of these YopH inhibitors showed low PIs (Supplementary data S1). For example, compound **2** was tested in 551 assays and was active in just two assays (pyruvate kinase and EBI2 receptor). Compound **7** was only active in 8 of 570 assays. The results suggest that these YopH inhibitors are unlikely to be promiscuous, though this requires further evaluation. In contrast, Compounds **15** and **16** showed an unusual high PI (active in 134 of 560 assays and 6 of 11 assays, respectively), indicating that the core of pyrimidotriazine-dione is probably a promiscuous one.

One of our goals in this study is to identify novel pTyr mimetics and functional groups favorable to the different binding sites of YopH for structure-based design of novel multi-valent inhibitors using fragment linking.^{22,23} As illustrated in the binding mode analysis of our identified compounds, an efficient strategy for developing YopH inhibitors is to target the conserved catalytic pocket with the FDR motif and the secondary phosphate binding site to improve the potency as well as selectivity. Figure 2 lists the pTyr surrogates and fragments that frequently scored best in docking and which possessed appreciable inhibitory activities against YopH. Some of the cyclic pTyr mimetics appear to be novel to YopH, whereas the fragments identified at the secondary phosphate binding site are likely more selective. It is worth mentioning that most of the pTyr mimetics and functional groups were able to bind to the FDR site, therefore, could be utilized to target both sites by tethering to a common connector such as a heterocyclic ring of furan and pyrrole.

In summary, using high throughput and virtual screening approaches we identified a number of potent inhibitors against the *Yersinia* virulence effector YopH. These small molecule compounds represent a diversity of chemotypes and pTyr mimetics. Characterization of the binding modes of these inhibitors provides a useful guide for structure-based design of potent and selective inhibitor as drug candidates to combat *Yersinia* virulence.

Acknowledgments

This work was funded in part by Program Grant 1U19AI056510 from the National Institute of Allergy and Infectious Disease and research funds from the Rockefeller University.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmcl.2012.12.018>.

References and notes

1. Brubaker, R. R. *Clin. Microbiol. Rev.* **1991**, *4*, 309.
2. Hinnebusch, B. J. *J. Mol. Med. (Berl.)* **1997**, *75*, 645.
3. Inglesby, T. V.; Dennis, D. T.; Henderson, D. A.; Bartlett, J. G.; Ascher, M. S.; Eitzen, E.; Fine, A. D.; Friedlander, A. M.; Hauer, J.; Koerner, J. F.; Layton, M.; McDade, J.; Osterholm, M. T.; O'Toole, T.; Parker, G.; Perl, T. M.; Russell, P. K.; Schoch-Spana, M.; Tonat, K. *JAMA* **2000**, *283*, 2281.
4. Cornelis, G. R.; Van Gijsegem, F. *Annu. Rev. Microbiol.* **2000**, *54*, 735.
5. Guan, K. L.; Dixon, J. E. *Science* **1990**, *249*, 553.
6. Chen, Y. T.; Seto, C. T. *J. Med. Chem.* **2002**, *45*, 3946.
7. Liang, F.; Huang, Z.; Lee, S. Y.; Liang, J.; Ivanov, M. I.; Alonso, A.; Bliska, J. B.; Lawrence, D. S.; Mustelin, T.; Zhang, Z. Y. *J. Biol. Chem.* **2003**, *278*, 41734.
8. Xie, J.; Comeau, A. B.; Seto, C. T. *Org. Lett.* **2004**, *6*, 83.
9. Huang, Z.; He, Y.; Zhang, X.; Gunawan, A.; Wu, L.; Zhang, Z. Y.; Wong, C. F. *Chem. Biol. Drug Des.* **2010**, *76*, 85.
10. Leone, M.; Barile, E.; Vazquez, J.; Mei, A.; Guiney, D.; Dahl, R.; Pellecchia, M. *Chem. Biol. Drug Des.* **2010**, *76*, 10.
11. Liu, F.; Hakami, R. M.; Dyas, B.; Bahta, M.; Lountos, G. T.; Waugh, D. S.; Ulrich, R. G.; Burke, T. R., Jr. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 2813.
12. Tautz, L.; Bruckner, S.; Sareth, S.; Alonso, A.; Bogetz, J.; Bottini, N.; Pellecchia, M.; Mustelin, T. *J. Biol. Chem.* **2005**, *280*, 9400.
13. Phan, J.; Lee, K.; Cherry, S.; Tropea, J. E.; Burke, T. R., Jr.; Waugh, D. S. *Biochemistry* **2003**, *42*, 13113.
14. Sun, J. P.; Wu, L.; Fedorov, A. A.; Almo, S. C.; Zhang, Z. Y. *J. Biol. Chem.* **2003**, *278*, 33392.
15. Kim, S. E.; Bahta, M.; Lountos, G. T.; Ulrich, R. G.; Burke, T. R., Jr.; Waugh, D. S. *Acta Crystallogr. D Biol. Crystallogr.* **2011**, *67*, 639.
16. Bahta, M.; Lountos, G. T.; Dyas, B.; Kim, S. E.; Ulrich, R. G.; Waugh, D. S.; Burke, T. R., Jr. *J. Med. Chem.* **2011**, *54*, 2933.
17. Hu, X.; Prehna, G.; Stebbins, C. E. *J. Med. Chem.* **2007**, *50*, 3980.
18. Hu, X.; Vujanac, M.; Stebbins, C. E. *J. Mol. Graph Model.* **2004**, *23*, 175.
19. Hu, X.; Stebbins, C. E. *Bioorg. Med. Chem.* **2005**, *13*, 1101.
20. Szczepankiewicz, B. G.; Liu, G.; Hajduk, P. J.; Abad-Zapatero, C.; Pei, Z.; Xin, Z.; Lubben, T. H.; Trevillyan, J. M.; Stashko, M. A.; Ballaron, S. J.; Liang, H.; Huang, F.; Hutchins, C. W.; Fesik, S. W.; Jirousek, M. R. *JACS* **2003**, *125*, 4087.
21. Hu, X.; Legler, P. M.; Khavrutskii, I.; Scorpio, A.; Compton, J. R.; Robertson, K. L.; Friedlander, A. M.; Wallqvist, A. *Biochemistry* **2012**, *51*, 1199.
22. Xie, J.; Seto, C. T. *Bioorg. Med. Chem.* **2005**, *13*, 2981.
23. Comeau, A. B.; Critton, D. A.; Page, R.; Seto, C. T. *J. Med. Chem.* **2010**, *53*, 6768.